Bioevolution of Chitosan and It's quaternary derivatives

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

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A Satyam Roychowdhury initiative

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Kolkata

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-: To whom it may concern :-

This is to certify that the thesis entitled "**Bioevolution of chitosan and it's quaternary derivatives**", which is submitted by Ms. Madhumita Haldar, in partial fulfilment of the requirement for the award of M.Sc. degree in Microbiology, at Sister Nivedita University, is a record of the candidate's own original work, carried out under my supervision. The matter embodied in this dissertation has not been submitted elsewhere before for the award of a degree or diploma. Madhumita is a diligent student. I wish her every success in life.

04/06/2025

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "Chitosan and It's quaternary derivatives" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from 4th January, 2025 to 2nd June,2025 under the supervision of Dr. Sandipan Chatterjee Department of Central Leather Research Institute, Regional Centre, Kolkata.

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled "Bioevolution of Chitosan and It's Quaternary Derivatives", submitted by Ms. Madhumita Halder (Registration No. 230010293011 Enrolment No. 2322211001029) to the Department of Microbiology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

Date: 03.06.2025

Place: Kolkata

Signature of Head of the Department Seal

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Madhumita Halder

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Abbreviations	COS: Chitosan oligosaccharide, TMC: N-N-N-Trimethyl chitosan, CMC: Craboxymethyl chitosan, NaOH: Sodium hydroxide, HCl: Hydrochloric acid, RBCs: Red blood cells, PBS: Phosphate buffer saline, PBMC: Peripheral blood mononuclear cells, DPPH: 2,2-Diphenyl-1-Picrylhydrazyl, MTT assay: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), NMR: Nuclear Magnetic Resonance, DMSO: Dimethyl sμLfoxide, EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS: N-hydroxysuccinimide.	

Abstract

Chitosan is a natural cationic biopolymer derived from the alkaline hydrolysis of chitin. It is composed of N-acetyl-D-glucosamine and D-glucosamine, and shows antibacterial, antitumour, haemostatic, wound healing and other biological activities, while also being biodegradable, biocompatible, blood compatible, and non-toxic. The two facets of its advantages make it a compelling material for medical applications. Even through its hydroxyl and amino active groups limits its solubilisation in alkaline solutions and most organic solvents, the chemical amenability of chitosan has proven to be of value in overcoming this problem. The modifications improving the performance of Chitosan have been extensively studied and is an active field of research owing to its potential applications. Modified chitosan derivatives have better biocompatibility, bioactivity, biodegradability, and non-toxicity, and they still possess the original bactericidal, antibacterial, anticancer, and antiviral pharmacological effects, including the ability to induce erythrocyte aggregation, promote platelet activation, and activate complement systems other than that of chitosan (Christou et al., Iftime et al.) One of the derivatives of Chitosan is Chitosan Oligosaccharide (COS), which shows excellent solubility in water in normal pH and is more bioavailable than unmodified chitosan (Muanprasat et al.). Though modifying the chitosan is a practice of priority, reports on modifications of chitosan oligosaccharide is not abundant. Modifications focusing on COS rather than Chitosan itself is intended to make downstream processing less labour intensive, as well as retaining the characteristics of superior solubility while introducing modifications aimed at specific applications. Our study aims to check if the modification done on Chitosan Oligosaccharide retains the biological activities of modified chitosan. This study can provide an establishment upon which further investigations can be built upon. The chitosan is obtained from the shells of Indian red mud crab (*Scylla serrata*) and is further hydrolysed to make chitosan oligosaccharides, which is further trimethylated to make a distinct derivative. The derivatives thus synthesized is subjected to assays to determine their effects on mammalian cells, pathogens, and their antioxidant activities.

Keywords: COS, CMC, TMC, Carnitine, Cytotoxicity, haemocompatibility, NMR

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Bio evolution of Chitosan and Its quaternary derivatives

Chitosan, a linear polysaccharide derived from the de-acetylation of chitin, has emerged as a highly versatile biopolymer owing to its biodegradability, biocompatibility, and functional properties. Its application spans across biomedical, agricultural, pharmaceutical, and environmental domains, primarily due to its unique chemical structure and modifiability. Derived predominantly from crustacean shells, chitosan has undergone an evolutionary leap throμgh chemical and enzymatic modifications, leading to the development of several high-performance derivatives such as Chitosan Oligosaccharide (COS), N,N,N-trimethyl chitosan (TMC), Carboxymethyl Chitosan (CMC), and the less explored Carnitine.

The concept of bio evolution in this context refers not to the evolutionary biology of chitosan in living organisms, but rather its transformation and enhancement throμgh bioengineering and chemical innovation over decades. These developments have amplified chitosan's properties and tailored them for specific biomedical and pharmaceutical needs.

Historical Context and Origin

Chitosan's origin lies in chitin, the second most abundant polysaccharide after cellμLose. Chitin is primarily sourced from the exoskeletons of crustaceans like shrimp and crabs and throμgh deacetylation processes involving alkali treatment, it is converted into chitosan. Traditional extraction methods have been extensively modified to yield purer, more reactive forms of chitosan.

Table 1: Evolutionary Timeline of Chitosan Derivatives

Year	Development	Key Feature
1859	Chitosan isolated	First recorded discovery
1980s	Biomedical use explored	Antimicrobial and biocompatibility tested
1997	TMC synthesised	Improved solubility and absorption
2003	CMC advancement	Enhanced water solubility, drμg delivery potential
2020s	Carnitine studies	Enhanced receptor interaction, novel applications

MolecμLar and Structural Basis

Chitosan is composed of β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units. The degree of deacetylation (DDA), molecμLar weight, and pH conditions influence its solubility and interaction with biological systems. Modifications typically target the amino and hydroxyl groups of the glucosamine backbone.

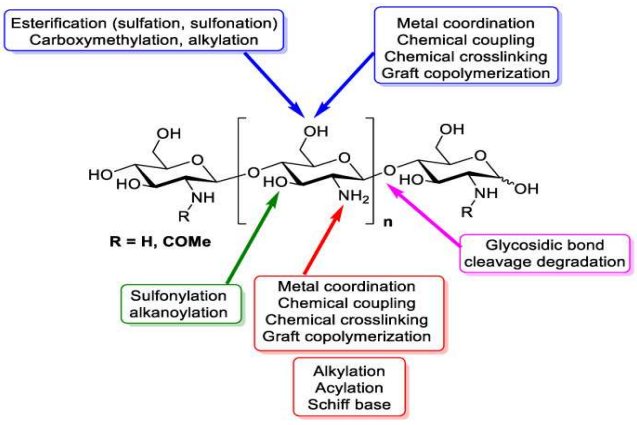

Figure 1: *Basic chemical structure of chitosan and its reactive sites.*

Relevance and Rationale for Derivative Development

The need for chitosan derivatives stems from the limitations of native chitosan, especially its solubility in neutral and basic media and inconsistent bioactivity vary from one source to another. Modifications are done to lessen the molecular weight leading to molecules with better solubility and pharmacokinetics, improved targeting, and enhanced biocompatibility.

Structural Characteristics of Chitosan and Its Derivatives

Chitosan is a natural polysaccharide derived via alkaline deacetylation of chitin, composed predominantly of β-(1-4)-linked D-glucosamine units, with varying degrees of N-acetyl-D-glucosamine depending on the extent of deacetylation (Chatterjee et al., 2003; Chen et al., 2022). Its basic repeating unit consists of a glucosamine monomer with a free amine group (-NH_2) at the C2 position. This reactive amine is the cornerstone of chitosan's chemical versatility, enabling a multitude of modifications yielding derivatives with tailored solubility, charge, and bioactivity (Bakshi et al., 2020).

MATERIALS AND METHODS

The chitosan sample, prepared from red mud crab (*Scylla serrata*), was provided by CSIR CLRI for deriving chitosan oligosaccharide and its derivative. Chemicals were provided by CSIR-CLRI. Biological assays and experiments were carried out at CSIR NEERI and CSIR IICB TRUE Campus under Dr. R. Jain and Dr. S. Kumar respectively.

Native Chitosan Structure

Chitosan is a semi-crystalline polymer whose insolubility at neutral pH limits its pharmaceutical utility. However, the primary amine groups confer cationic properties under acidic conditions, making chitosan uniquely bioadhesive and reactive toward poly-anionic compounds (Douglas de Britto & Assis, 2007). Unmodified chitosan exhibits a high degree of hydrogen bonding due to hydroxyl and amino groups, resulting in poor solubility and limited aqueous purposes. This Chitosan is prepared by Hackman method in which the crab shells were dried and pulverised, and then treated with 2 N HCl at 0°C for 48 hours while stirring. It was then washed with water and treated with 50% NaOH for 7 hours, at 120°C under N_2 atmosphere. The resulting mass was washed with water and triturated with warm acetone, dried and preserved (S. Chatterjee et al.).

Chitosan Oligosaccharides (COS)

Chitosan oligosaccharides are low-molecular-weight fragments of chitosan, generally composed of 2–10 glucosamine units. Due to their reduced polymerisation, COS are significantly more soluble in water across a broad pH range. Their small size enhances their permeability and bioavailability, making them ideal candidates for drµg delivery and antioxidant applications (Chen et al., 2022; Mourya et al., 2010).

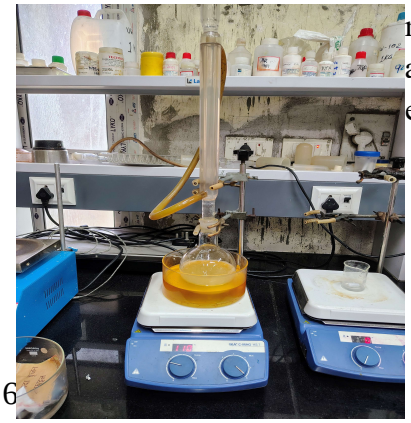

Figure 2.1: Triturated

Figure 2.2: Precipitated

Figure 2.3: Filtred

Figure 2.4: Lyophilized

1g of Chitosan was dissolved in 100 mL of 5% HCl solution for 6 Hrs. at 90 degree Celsius in reflux condition. The pH of the solution was changed into 5.5 by 2N NaOH and precipitated by 1:1(v/v) of Ethanol and Acetone in the next step. The precipitation was then filtered out on Whatman filter paper, dialysed into thin dialysis membrane for 3 days and lyophilised at -42 degree Celsius to get dry powder of Chitosan Oligosaccharide. .

N,N,N-Trimethyl Chitosan (TMC)

TMC is a quaternized derivative synthesised via methylation of the chitosan's amine group, yielding permanent positive charges regardless of the medium's pH (Polnok et al., 2004; Kotzé et al., 1999). This significantly improves aqueous solubility, especially under physiological conditions, and enhances interaction with negatively charged cell membranes and tight junction proteins. The presence of trimethylated nitrogen centres introduces steric hindrance, reducing chain aggregation and increasing water interaction. TMC is a leading candidate for mucosal vaccine delivery due to these mucoadhesive and permeability-enhancing properties (Illum et al., 2001; Boontha et al., 2008).

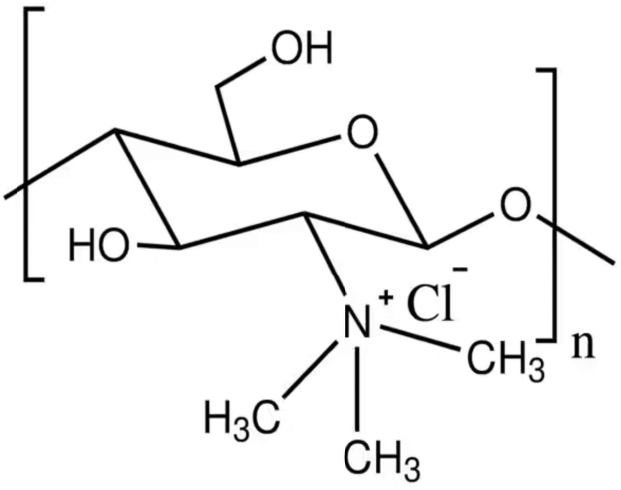

Figure3 1: N,N,N-Trimethyl Chitosan (TMC)

To prepare N,N,N-Trimethyl Chitosan (TMC), 1 gram of hydrolysed chitosan was taken in a round bottom flask. To it, 16 mL of dimethyl sulfate and 4 mL of water was added. 1.2 grams of NaOH and 0.88 grams of NaCl was added to the solution and mixed for 6 hours at room temperature. The final product was precipitated using acetone, dialysed, vacuum dried, and collected after lyophilisation. (Britto et al.)

Carboxymethyl Chitosan (CMC)

Carboxymethylation of chitosan introduces carboxylic groups onto either the amine or hydroxyl sites, significantly improving solubility at neutral and basic pH levels (Mourya et al., 2010). This amphoteric nature allows CMC to function as a pH-responsive polymer with applications in wound healing and pH-sensitive drµg delivery systems (Kato et al., 2003). The substitution degree and position (N- or O-carboxymethylation) govern its physicochemical behaviour, including viscosity, charge distribution, and gelling properties (Bakshi et al., 2020; Iftime et al., 2019).

CMC synthesis- 1 g of Chitosan oligosaccharide was dissolved in 20 mL 20% NaOH and stirred for 15 mins. 3 grams of chloroacetic acid was added into 25 mL of ethanol and this mixture was slowly poured into the COS-NaOH mixture. It is then kept at 40 degree Celsius for overnight. The next step is to treat it with 10% glacial acetic acid in order to neutralise the pH. The precipitation was collected by adding 300 mL of ethanol with it. The solution was then filtered by Whatman filter paper and the precipitate was dialysed, lyophilised to obtain CMC powder.

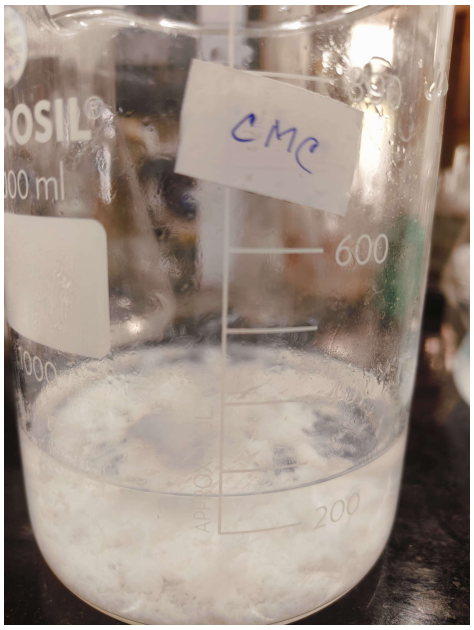

Figure 3.1: Carboxymethyl Chitosan

Figure 3.2: Precipitate of CMC

Carnitine

Althoµgh not widely documented, *carnitine* is proposed to be an amphiphilic derivative incorporating long-chain hydrophobic substitutions (hypothetically C8–C12 alkyl chains) to chitosan's hydroxyl or amino sites. This structural motif imparts surfactant-like behaviour and may be ideal for micelle formation or lipid interaction in nanoemulsion systems (Zhang et al., 2017; Davis, 2001).

Carnitine Synthesis- 2.6 mM of L-carnitine, 5.2 mM of EDC and 5.2 mM of NHS were dissolved into 30 mL of ethanol at 60 degree Celsius for 30 mins. 1 g of COS was dissolved in 100 mL of distilled water. The dissolved chitosan was then mixed with the reaction mixture which altogether were kept at 60 degree Celsius in reflux condition for overnight. The solution is precipitated with 300 mL of acetone, dialysed and lyophilised.

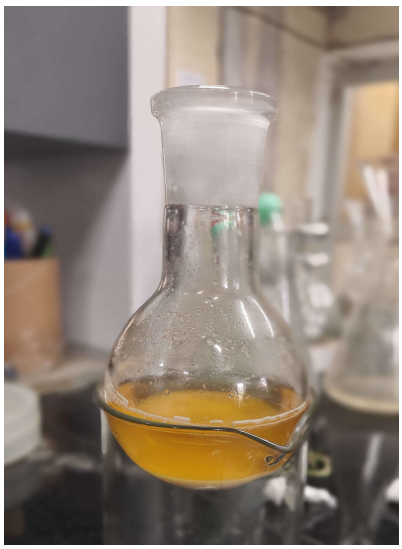

Figure 4.1: solution of Cirnitine

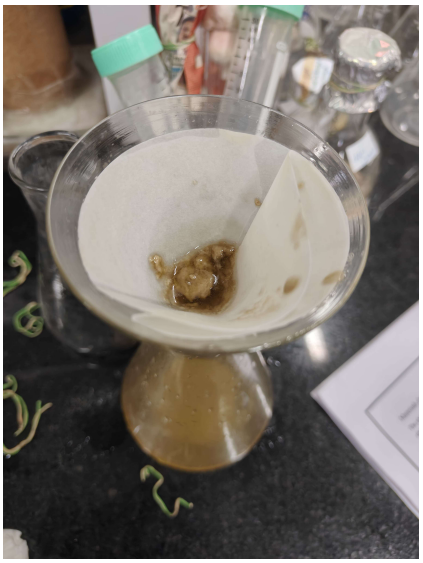

Figure4.2: precipitate of Cirnitine

Table2 – Structural Insights

Derivative	Key Functional Groups	Solubility (Neutral pH)	Charge Behaviour	Application Focus
Chitosan	–NH_2, –OH	Low	pH-dependent (+)	Film, scaffold
COS	–NH_2, –OH	High	pH-dependent (+)	Antioxidant, drµg delivery
TMC	–$N^+(CH_3)_3$	Very High	Permanent (+)	Nasal, vaccine adjuvant
CMC	–COOH, –NH_2	Moderate to high	Amphoteric	Wound healing, pH response
Carnitine	–NH_2, Alkyl chains	Low	Amphiphilic	EmµLsions, nanocarriers

Experimental Principles and Interpretations

This chapter provides a comprehensive breakdown of the experimental techniques used to characterise chitosan and its derivatives. Each assay sheds light on different functional properties, critical for biomedical and pharmaceutical applications.

1. Antimicrobial Activity Assay

Principle:

Chitosan and its derivatives exhibit antimicrobial activity primarily due to their **poly-cationic nature**, which disrupts microbial cell membranes. The mechanism involves: Electrostatic interaction between positively charged amino groups and negatively charged microbial surfaces, binding to DNA, thus inhibiting replication and transcription (Bakshi et al., 2020; Chatterjee et al., 2003).

The bacteria *Staphylococcus aureus, Escherichia coli* were first cultured in nutrient broth for 24 hours. After incubation, 100 µ of the culture was spread plated on nutrient agar medium. Antibiotic discs of 30, 50 & 75 mg/mL of Chitosan and Its quaternary derivatives were prepared. Cultured

media with different bacterial strain were incubated along with the antibiotic discs. After 24 hours of incubation, the inhibition zones were measured.

Interpretation:

- TMC shows superior activity due to permanent quaternary ammonium groups.

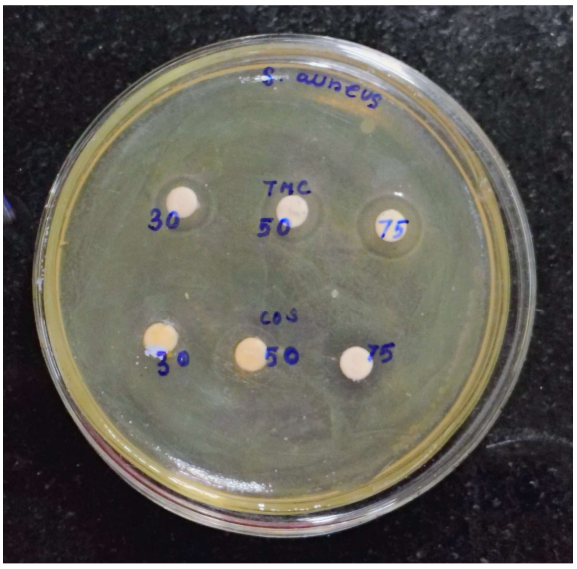

Figure 5.1: MIC on S.aureus

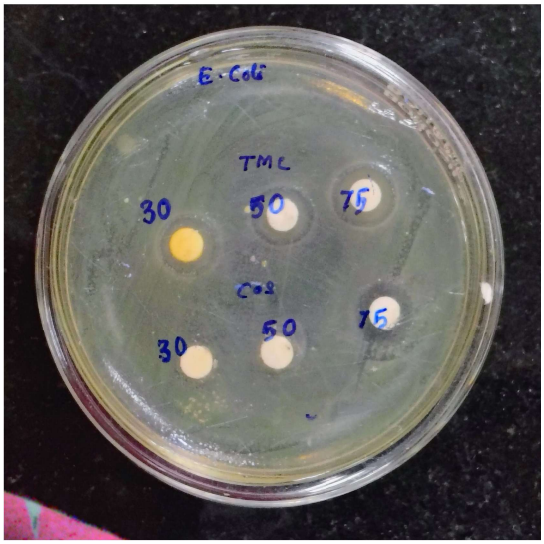

Figure 5.2: MIC on E.coli

- COS demonstrates moderate activity due to smaller size and rapid membrane penetration.

2. Haemocompatibility Assay

The haemolysis assay evaluates the biocompatibility of chitosan derivatives with red blood cells (RBCs). It measures the percentage of RBCs degraded upon exposure to the polymer. Human RBCs were separated from plasma by centrifuging them for 10 mins at 4,500 RPM and washed thrice subsequently with PBS saline buffer. RBCs are incubated with polymer solutions at various concentrations (5-20 mg/mL) taking TitronX100 as control (0.1 mg/mL). Absorbance at 540 nm used to quantify haemoglobin release.

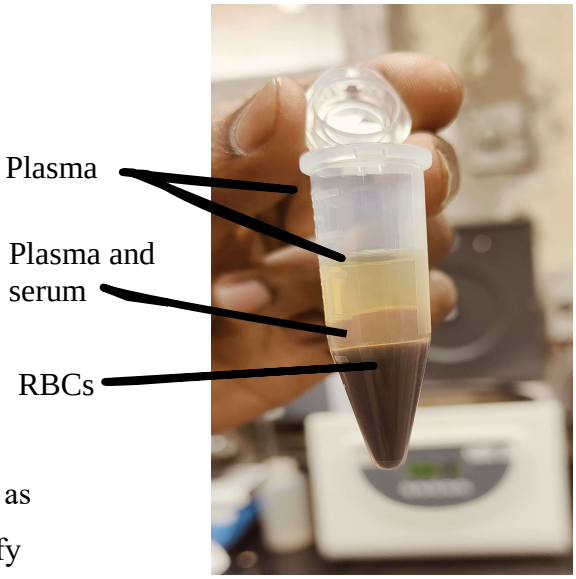

Figure 6: separating RBCs from plasma

Table3

Haemolysis (%) of different chitosan derivatives:

Derivatives	Hemocompatible (%)			
	5mg	**10 mg**	**15mg**	**20mg**
COS	85%	81%	69%	43.75%
TMC	88%	87%	83%	81%
Carnitine	91%	91.9%	90%	87.5%

3. DPPH Free Radical Scavenging Assay

The DPPH assay measures antioxidant activity by evaluating the polymer's ability to donate hydrogen to neutralise the DPPH free radical (purple → yellow). 0.1 mM of DPPH solution was prepared in ethanol. Blank was made by mixing DPPH solution, Ethanol and distilled water in 1:1:1 (v/v/v) ratio, a positive control was made by mixing DPPH solution, Ethanol, distilled water and 10 mg/mL Ascorbic acid in 1:1:1:1 (v/v/v/v) ratio, test mixtures were made by keeping all the component in the later mixture excluded Ascorbic acid, included Chitosan and Its quaternary derivatives in the concentrations of 5mg/mL, 10mg/mL, 15mg/mL, 20mg/mL, 25mg/mL. After 30-min incubation with polymer solution absorbance were measured at 517 nm measured.

Scavenging activity (%) = (1- (Absorbance of Sample / Absorbance of Control)) * 100

Table 4

DPPH Scavenging Activity:

Derivative	Absorbance at 517nm				
	5mg/mL	**10mg/mL**	**15mg/mL**	**20mg/mL**	**25mg/mL**
COS	0.62	0.6	0.58	0.55	0.54
TMC	0.61	0.54	0.5	0.43	0.4

4. Cytotoxicity on PBMC Cell Line

The MTT assay was performed to assess the cytotoxicity of samples COS and TMC. In short, 5000/ well-htert-rpe and HeLa cells were seeded in 96-well plates and incubated in the incubator at 5% CO_2 concentration in a CO_2 incubator to allow cells to stick and reach the desired confluency (70-80%). After incubation, treatment was performed by COS and TMC at various sample concentratio ns and incubated for 24 hours. The MTT solution was produced by PBS-buffer MTT powder at a concentration of 5 mg/ml. The media is discarded. The final concentration of MTT shoμLd be about 0.5 mg/ml. Plates were incubated at 37°C in 5% CO_2 for 4 hours.

Incubation showed that the plates were carefμLly removed to avoid disturbing the crystals of the shape. 100μL from DMSO was given each deepening to remove formal crystals. The plates were then incubated at room temperature for 10-15 min, gently shaking to ensure complete solubilisation. Absorption was then measured with a 570 nm by an UV-Spectrophotometer.

Structural Characterisation and Chemical Behaviour of Chitosan Derivatives

Chitosan and its derivatives possess distinct structural and physicochemical characteristics that influence their solubility, biological interactions, and functional versatility. The incorporation of specific chemical groups alters hydrogen bonding, electrostatic interactions, and molecular conformation, which can be visualised through NMR.

NMR Analysis

1. COS shows reduced peak complexity, with signals clustered in the 3.0-4.0 ppm region that represent the protons attached to the sμgar rings of the chitosan backbone. Chitosan oligosaccharides consist of repeating glucosamine units, so these peaks are likely associated with the hydrogen atoms on carbons adjacent to hydroxyl and amine groups, which appear in this region. The signal at 3.09 ppm likely comes from the protons attached to the amine groups in the glucosamine units of chitosan. The peak at 1.97 ppm is possibly related to the acetyl group in the N-acetylglucosamine residues. Chitosan oligosaccharide is partially deacetylated, and this signal would represent the methyl protons in any remaining acetyl groups. The broad peaks around 3.7-3.9 ppm sμggests overlapping signals from various protons on the sμgar rings, which is common for polysaccharides. The relatively sharp peaks indicate well-defined molecular environments, which might correspond to the repeating units of the oligosaccharide. In conclusion, the desired product has been synthesised.

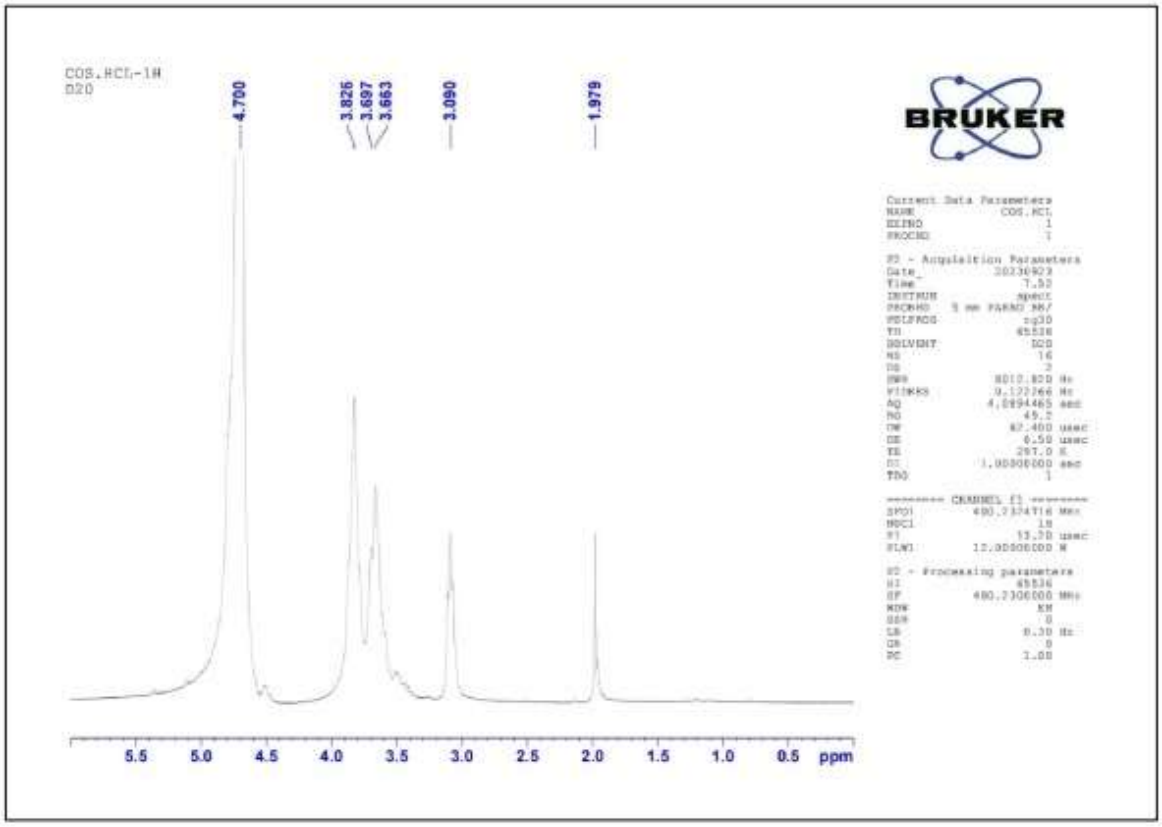

Figure 7.1: NMR sample of COS

2. The peak at 3.23 ppm corresponds to the protons of the N-N-N- trimethyl ammonium groups attached to the nitrogen atoms. The sharp signal is characteristic if the methyl protons in these groups. The chemical shift is slightly above 3 ppm, consistent with other methylated nitrogen environments. There are also signal between 3.6-3.8, which represents the protons on the glucosamine units. These are similar to the unmodified chitosan oligosaccharide, as the sµgar backbone remains largely unchanged by the trimethylation process. The multiplets around this region are due to overlapping signals from different protons in the glucosamine units. The signal at 3.09 ppm in the spectrum of chitosan oligosaccharide is missing in this spectrum, which is consistent with the fact that the nitrogen atoms are now fully substituted with methyl groups, eliminating NH protons. The peak at 1.93 ppm likely corresponds to the acetyl group on any remaining N-acetylglucosamine residues, typical of the methyl group attached to the nitrogen in acetyl groups.

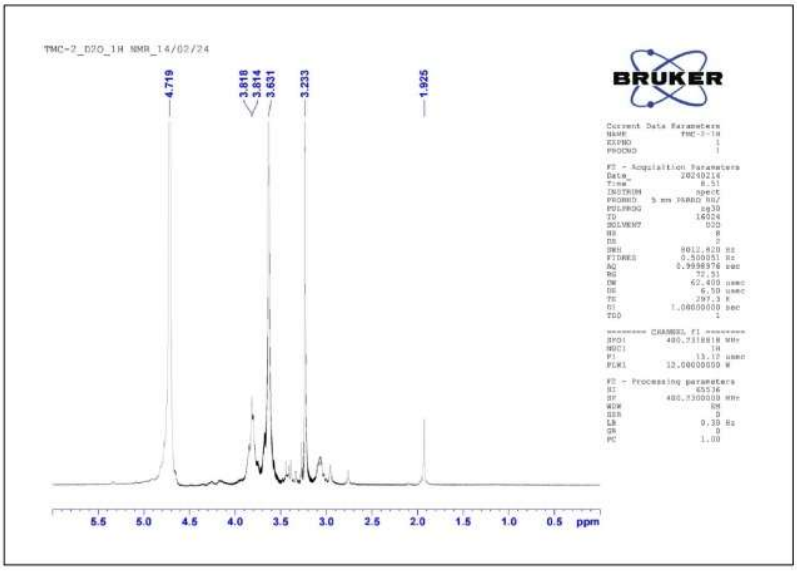

Figure 7.2: NMR sample of TMC

Comparative Biological Effects

1. cytotoxicity studies reinforce the safety of these derivatives in biomedical applications (Bakshi et al., 2020; Ghasemi et al., 2021; Illum et al., 1998). Almost 100 percent viable cell are present upto 500 microgram per mL concentrations. Which makes it wonderfully biocompatible.

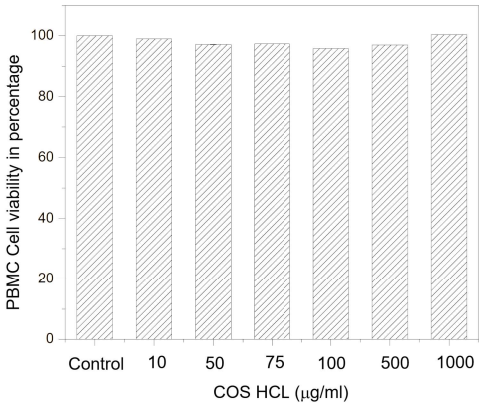

Figure 8.1: Cytotoxicity measurement of COS

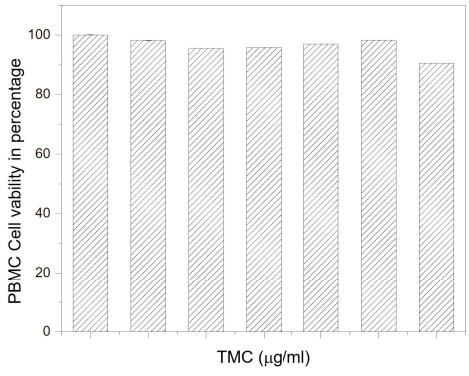

Figure 8.2: Cytotoxicity measurement of TMC

2. Haemolysis: Chitosan and COS are hemocompatible (<5% haemolysis). Carnitine with its long amphoteric chain shows a great hemocompatibility at 15 mg/mL which is almost 70%.

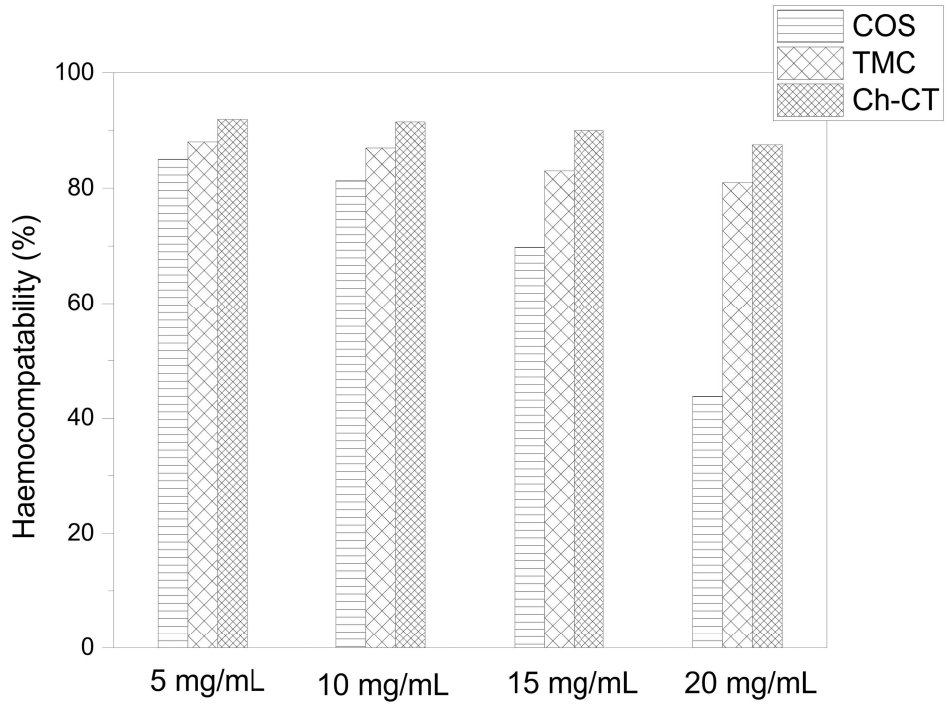

Figure 9: Comaparative analysis of hemocompatibility of Chitosan and it's quaternary derivatives

3. DPPH Scavenging: COS shows the great antioxidant activity due to available free amino groups, TMC exhibit more activity depending on substitution patterns. The short chain length and high solubility contributes significantly to the antioxidant activity of COS. The primary amino groups and hydroxyl groups can donate electrons or hydrogen to neutralise free radicals; in this case DPPH. Despite its lack of primary amines which are replaced by quaternary amines, TMC still can scavenge free radicals due to the presence of hydroxyl groups.

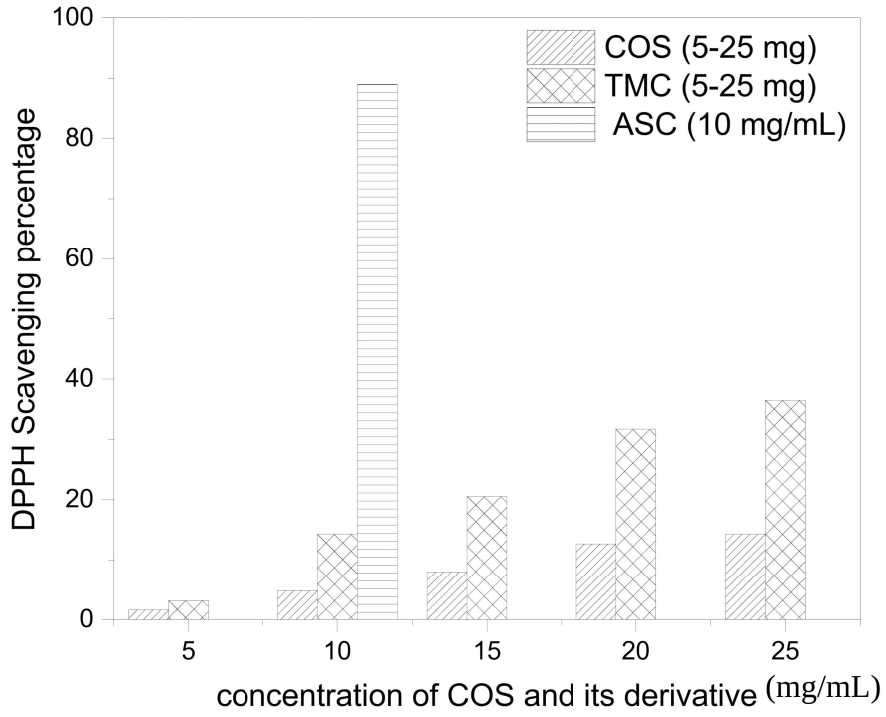

Figure 10: Comaparative analysis of DPPH sacavanging of Chitosan and it's quaternary derivatives

Conclusion and Future Prospects

Chitosan and its chemically modified derivatives present a remarkable array of bioactivities and physicochemical behaviours that suit diverse biomedical and industrial applications. This thesis illustrated that structural fine-tuning through methylation, carboxylation, or depolymerization that can significantly enhance the solubility, bioavailability, and functional performance of chitosan. Chitosan oligosaccharide is a promising low-toxicity antioxidant, TMC shows exceptional antimicrobial and drμg delivery potential, CMC excels in hydrogel and scaffold applications, Structure-function relationships dictate application-specific optimisation which are targeting nanoparticle formulations using TMC for mucosal vaccines, developing smart hydrogels using CMC with pH-responsiveness, investigating synergistic combinations of COS and other biopolymers for regenerative medicine.

Recent advancements in the synthesis of chitosan oligosaccharides (COS) have highlighted the potential of hydrolysing chitosan into oligosaccharides before further modification. This approach aligns with traditional methods of direct chitosan modification and offers a promising foundation for enhancing chitosan-derived products.

To optimise COS production, various methods have been explored. Chemical hydrolysis, particularly using acids like hydrochloric acid, is commonly employed at an industrial scale due to its cost-effectiveness. However, this method often results in low yields and a mixture of products

with varying degrees of polymerisation and acetylation, posing challenges for purification and consistency.

Enzymatic hydrolysis presents an alternative, utilising specific enzymes like chitosanases to break down chitosan into COS. This method offers advantages such as mild reaction conditions, high selectivity, and reduced environmental impact. Nevertheless, the high cost and limited availability of specific enzymes can hinder large-scale industrial application.

Additionally, physical methods such as microwave-assisted extraction and ultrasonic treatment have been explored for their potential to improve efficiency and sustainability in COS production. These techniques can decrease reaction times and energy consumption, contributing to more sustainable production processes.

In summary, while the hydrolysis of chitosan into oligosaccharides followed by modification is a promising strategy, ongoing research is essential to optimise these processes for economic viability and environmental sustainability. Future studies should focus on integrating these methods to develop scalable and cost-effective production techniques for chitosan-derived products.

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Abstract

Chitosan is a natural cationic biopolymer derived **from the** alkaline hydrolysis **of chitin** . It **is composed of N-acetyl-D-glucosamine and D-glucosamine** 2

, and shows antibacterial, antitumour, haemostatic, wound healing and other biological activities, while also being biodegradable, biocompatible, blood compatible, and non-toxic. The two facets of its advantages make it a compelling material for medical applications. Even thoμgh its hydroxyl and amino active groups limits its solubilisation in alkaline solutions and most organic solvents, the chemical amenability of chitosan has proven to be of value in overcoming this problem. The modifications improving the performance of Chitosan have been extensively studied and is an active field of research owing to its potential applications.

Modified chitosan derivatives have better biocompatibility, bioactivity, biodegradability, and non-toxicity, and they still possess the original bactericidal, antibacterial, anticancer, and antiviral pharmacological effects, including the ability to induce erythrocyte aggregation, promote platelet activation, and activate complement systems other than that of chitosan 1

(Christou et al., Iftime et al.) One of the derivatives of Chitosan is Chitosan Oligosaccharide (COS), which shows excellent solubility in water in normal pH and is more bioavailable than unmodified chitosan (Muanprasat et al.). Thoμgh modifying the chitosan is a practice of priority, reports on modifications of chitosan oligosaccharide is not abundant. Modifications focusing on COS rather than Chitosan itself is intended to make downstream processing less labour intensive, as well as retaining the characteristics of superior solubility while introducing modifications aimed at specific applications. Our study aims to check if the modification done on Chitosan Oligosaccharide retains the biological activities of modified chitosan. This study can provide an establishment upon which further investigations can be built upon. The chitosan is obtained from the shells of Indian red mud crab (Scylla serrata) and is further hydrolysed to make chitosan oligosaccharides, which is further trimethylated to make a distinct derivative. The derivatives thus synthesized is subjected to assays to determine their effects on mammalian cells, pathogens, and their antioxidant activities.

Keywords: COS, CMC, TMC, Carnitine, Cytotoxicity, haemocompatibility, NMR

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Timeline of Chitosan Derivatives.....5 MolecµLar and Structural Basis.....5 Relevance and Rationale for Derivative Development.....6 MATERIALS AND METHODS.....6 Native Chitosan Structure.....6 Chitosan Oligosaccharides (COS).....7 N,N,N-Trimethyl Chitosan (TMC).....7 Carboxymethyl Chitosan (CMC).....8 Carnitine..... .....8 Table2 – Structural Insights.....9 Experimental Principles and Interpretations.....9 1. Antimicrobial Activity Assay.....10 2. Hemocompatibility Assay.....10 The haemolysis assay evaluates the biocompatibility of.....11 chitosan derivatives with red blood cells (RBCs).....11 It measures the percentage of RBCs degraded upon.....11 exposure to the polymer. Human RBCs were separated.....11 from plasma by centrifµging them for 10 mins at.....11 10,000 RPM and washed thrice subsequently with PBS.....11 saline buffer. RBCs are incubated with polymer solutions.....11 at various concentrations (5-20 mg/mL) taking TitronX100 as.....11 control (0.1 mg/mL). Absorbance at 540 nm used to quantify.....11 haemoglobin release.....11 3. DPPH Free Radical Scavenging Assay.....11 4. Cytotoxicity on PBMC Cell Line.....11 Structural Characterisation and Chemical Behaviour of Chitosan Derivatives.....12 NMR analysis..... .....13 Comparative Biological Effects.....13-14 Conclusion..... .....15-16 References..... .....17-18 Appendix..... .....19 4 Bio evolution of Chitosan and Its quaternary derivatives Chitosan, a linear polysaccharide derived from the de-acetylation of chitin, has emerged as a highly versatile biopolymer owing to its biodegradability, biocompatibility, and functional properties. Its application spans across biomedical, agricµLtural, pharmaceutical, and environmental domains, primarily due to its unique chemical structure and modifiability. Derived predominantly from crustacean shells, chitosan has undergone an evolutionary leap throµgh chemical and enzymatic modifications, leading to the development of several high-performance derivatives such as Chitosan Oligosaccharide (COS), N,N,N-trimethyl chitosan (TMC), Carboxymethyl Chitosan (CMC), and the less explored Carnitine. The concept of bio evolution in this context refers not to the evolutionary biology of chitosan in living organisms, but rather its transformation and enhancement throµgh bioengineering and chemical innovation over decades. These developments have amplified chitosan's properties and

tailored them for specific biomedical and pharmaceutical needs. Historical Context and Origin Chitosan's origin lies in chitin, the second most abundant polysaccharide after cellµLose. Chitin is primarily sourced from the exoskeletons of crustaceans like shrimp and crabs and throµgh deacetylation processes involving alkali treatment, it is converted into chitosan. Traditional extraction methods have been extensively modified to yield purer, more reactive forms of chitosan. Table 1: Evolutionary Timeline of Chitosan Derivatives Year Development Key Feature 1859 Chitosan isolated First recorded discovery 1980s Biomedical use explored Antimicrobial and biocompatibility tested 1997 TMC synthesised Improved solubility and absorption 2003 CMC advancement Enhanced water solubility, drµg delivery potential 2020s Carnitine studies Enhanced receptor interaction, novel applications MolecµLar and Structural Basis

Chitosan is composed of β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units. The 3 degree **of**

deacetylation (DDA), molecµLar weight, and pH conditions influence its solubility and interaction with biological systems. Modifications typically target the amino and hydroxyl groups of the glucosamine backbone. Figure 1: Basic chemical structure of chitosan and its reactive sites. 5 Relevance and Rationale for Derivative Development The need for chitosan derivatives stems from the limitations of native chitosan, especially its solubility in neutral and basic media and inconsistent bioactivity vary from one source to another. Modifications are done to lessen the molecµLar weight leading to molecµLes with better solubility and pharmacokinetics, improved targeting, and enhanced biocompatibility. Structural Characteristics of Chitosan and Its Derivatives Chitosan is a natural polysaccharide derived via alkaline deacetylation of chitin, composed predominantly of β-(1-4)-linked D-glucosamine units, with varying degrees of N-acetyl- Dglucosamine depending on the extent of deacetylation (Chatterjee et al., 2003; Chen et al., 2022). Its basic repeating unit consists of a glucosamine monomer with a free amine group (- NH_2) at the C2 position. This reactive amine is the cornerstone of chitosan's chemical versatility, enabling a mµLtitude of modifications yielding derivatives with tailored solubility, charge, and bioactivity (Bakshi et al., 2020). MATERIALS AND METHODS The chitosan sample, prepared from red mud crab (Scylla serrata), was provided by CSIR CLRI for deriving chitosan oligosaccharide and its derivative. Chemicals were provided by CSIR-CLRI. Biological assays and experiments were carried out at CSIR NEERI and CSIR IICB TRUE Campus under Dr. R. Jain and Dr. S. Kumar respectively. Native Chitosan Structure Chitosan is a semi-crystalline polymer whose insolubility at neutral pH limits its pharmaceutical utility. However, the primary amine groups confer cationic properties under acidic conditions, making chitosan uniquely bioadhesive and reactive toward poly-anionic compounds (Doµglas de Britto & Assis, 2007). Unmodified chitosan exhibits a high degree of hydrogen bonding due to hydroxyl and amino groups, resµLting in poor solubility and limited aqueous purposes. This Chitosan is prepared by Hackman method in which the crab shells were dried and pµLverised, and then treated with 2 N HCl at 0°C for 48 hours while stirring. It was then washed with water and treated with 50% NaOH for 7 hours, at 120°C under N2 atmosphere. The resµLting mass was washed with water and triturated with warm acetone, dried and preserved (S. Chatterjee et al.). Chitosan Oligosaccharides (COS) Chitosan oligosaccharides are low-molecµLar-weight fragments of chitosan, generally composed of 2–10 glucosamine units. Due to their reduced polymerisation, COS are significantly more soluble in water across a broad pH range. Their small size enhances their permeability and bioavailability, making them ideal candidates for drµg delivery and antioxidant applications (Chen et al., 2022; Mourya et al., 2010). 6 Figure 2.1: Triturated Figure 2.2: Precipitated 1g of Chitosan was dissolved in 100 mL of 5% HCl solution for 6 Hrs. at 90 degree Celsius in reflux

condition. The pH of the solution was changed into 5.5 by 2N NaOH and precipitated by 1:1(v/v) of Ethanol and Acetone in the next step. The precipitation was then filtered out on Whatman filter paper, dialysed into thin dialysis membrane for 3 days and lyophilised at -42 degree Celsius to get dry powder of Chitosan Oligosaccharide. . N,N,N-Trimethyl Chitosan (TMC) TMC is a quaternized derivative synthesised via methylation of the chitosan's amine group, yielding Boontha et al., 2008). To prepare N,N,N-Trimethyl Chitosan (TMC), 1 gram of hydrolysed chitosan was taken in a round bottom flask. To it, 16 mL of dimethyl sµLfate and 4 mL of water was added. 1.2 grams of NaOH and 0.88 grams of NaCl was added to the solution and mixed for 6 hours at room temperature. The final product was precipitated using acetone, dialysed, vacuum dried, and collected after lyophilisation. (Britto et al.) 7 Figure 2.4: Lyophilized Figure 2.3: Filtred Carboxymethyl Chitosan (CMC) Carboxymethylation of chitosan introduces carboxylic groups onto either the amine or hydroxyl sites, significantly improving solubility at neutral and basic pH levels (Mourya et al., 2010). This amphoteric nature allows CMC to function as a pH-responsive polymer with applications in wound healing and pH-sensitive drµg delivery systems (Kato et al., 2003). The substitution degree and position (N- or O-carboxymethylation) govern its physicochemical behaviour, including viscosity, charge distribution, and gelling properties (Bakshi et al., 2020; Iftime et al., 2019). CMC synthesis- 1 g of Chitosan oligosaccharide was dissolved in 20 mL 20% NaOH and stirred for 15 mins. 3 grams of chloroacetic acid was added into 25 mL of ethanol and this mixture was slowly poured into the COS-NaOH mixture. It is then kept at 40 degree Celsius for overnight. The next step is to treat it with 10% glacial acetic acid in order to neutralise the pH. The precipitation was collected by adding 300 mL of ethanol with it. The solution was then filtered by Whatman filter paper and the precipitate was dialysed, lyophilised to obtain CMC powder. Carnitine Althoµgh not widely documented, carnitine is proposed to be an amphiphilic derivative incorporating long-chain hydrophobic substitutions (hypothetically C8–C12 alkyl chains) to chitosan's hydroxyl or amino sites. This structural motif imparts surfactant-like behaviour and may be ideal for micelle formation or lipid interaction in nanoemµLsion systems (Zhang et al., 2017; Davis, 2001). Carnitine Synthesis- 2.6 mM of L-carnitine, 5.2 mM of EDC and 5.2 mM of NHS were dissolved into 30 mL of ethanol at 60 degree Celsius for 30 mins. 1 g of COS was dissolved in 100 mL of distilled water. The dissolved chitosan was then mixed with the reaction mixture which altogether were kept at 60 degree Celsius in reflux condition for overnight. The solution is precipitated with 300 mL of acetone, dialysed and lyophilised. 8 Figure 3.1: Carboxymethyl Chitosan Figure 3.2: Precipitate of CMC Table2 – Structural Insights Derivative Key Functional Groups Solubility (Neutral pH) Charge Behaviour Application Focus Chitosan $-NH_2$, –OH Low pH-dependent (+) Film, scaffold COS $-NH_2$, –OH High pH-dependent (+) Antioxidant, drµg delivery TMC $-N^+(CH_3)_3$ Very High Permanent (+) Nasal, vaccine adjuvant CMC –COOH, $-NH_2$ Moderate to high Amphoteric Wound healing, pH response Carnitine $-NH_2$, Alkyl chains Low Amphiphilic EmµLsions, nanocarriers Experimental Principles and Interpretations This chapter provides a comprehensive breakdown of the experimental techniques used to characterise chitosan and its derivatives. Each assay sheds light on different functional properties, critical for biomedical and pharmaceutical applications. 1. Antimicrobial Activity Assay Principle: Chitosan and its derivatives exhibit antimicrobial activity primarily due to their poly- cationic nature, which disrupts microbial cell membranes. The mechanism involves: Electrostatic interaction between positively charged amino groups and negatively charged microbial surfaces, binding to DNA, thus inhibiting replication and transcription (Bakshi et al., 2020; Chatterjee et al., 2003). The bacteria Staphylococcus aureus, Escherichia coli were first cµLtured in nutrient broth for 24 hours. After incubation, 100 µ of the cµLture was spread plated on nutrient agar medium. Antibiotic discs of 30, 50 & 75 mg/mL of Chitosan and Its quaternary derivatives were prepared. CµLtured 9 Figure 4.1: solution of Cirnitine Figure4.2: precipitate of Cirnitine media with different bacterial strain were incubated along with the antibiotic discs. After 24 hours of incubation, the inhibition zones were measured. Interpretation: • TMC shows superior activity due to

permanent quaternary ammonium groups. • COS demonstrates moderate activity due to smaller size and rapid membrane penetration. 2. Hemocompatibility Assay The haemolysis assay evaluates the biocompatibility of chitosan derivatives with red blood cells (RBCs). It measures the percentage of RBCs degraded upon exposure to the polymer. Human RBCs were separated from plasma by centrifµging them for 10 mins at 4,500 RPM and washed thrice subsequently with PBS saline buffer. RBCs are incubated with polymer solutions at various concentrations (5-20 mg/mL) taking TitronX100 as control (0.1 mg/mL). Absorbance at 540 nm used to quantify haemoglobin release. 10 Figure 6: separating RBCs from plasma Plasma Plasma and serum RBCs Table3 Haemolysis (%) of different chitosan derivatives: Derivatives Hemocompatible (%) 5mg 10 mg 15mg 20mg COS 85% 81% 69% 43.75% TMC 88% 87% 83% 81% Carnitine 91% 91.9% 90% 87.5% 3. DPPH Free Radical Scavenging Assay The DPPH assay measures antioxidant activity by evaluating the polymer's ability to donate hydrogen to neutralise the DPPH free radical (purple → yellow). 0.1 mM of DPPH solution was prepared in ethanol. Blank was made by mixing DPPH solution, Ethanol and distilled water in 1:1:1 (v/v/v) ratio, a positive control was made by mixing DPPH solution, Ethanol, distilled water and 10 mg/mL Ascorbic acid in 1:1:1:1 (v/v/v/v) ratio, test mixtures were made by keeping all the component in the later mixture excluded Ascorbic acid, included Chitosan and Its quaternary derivatives in the concentrations of 5mg/mL, 10mg/mL, 15mg/mL, 20mg/mL, 25mg/mL. After 30- min incubation with polymer solution absorbance were measured at 517 nm measured. Scavenging activity (%) = (1- (Absorbance of Sample / Absorbance of Control)) * 100 Table 4 DPPH Scavenging Activity: Derivative Absorbance at 517nm 5mg/mL 10mg/mL 15mg/mL 20mg/mL 25mg/mL COS 0.62 0.6 0.58 0.55 0.54 TMC 0.61 0.54 0.5 0.43 0.4 4. Cytotoxicity on PBMC Cell Line The MTT assay was performed to assess the cytotoxicity of samples COS and TMC. In short, 5000/ well-htert-rpe and HeLa cells were seeded in 96-well plates and incubated in the incubator at 5% CO2 concentration in a CO2 incubator to allow cells to stick and reach the desired confluency (70- 80%). After incubation, treatment was performed by COS and TMC at various sample concentratio ns and incubated for 24 hours. The MTT solution was produced by PBS-buffer MTT powder at a concentration of 5 mg/ml. The media is discarded. The final concentration of MTT shoµLd be about 0.5 mg/ml.

Plates were incubated at 37°C in **5% CO2 for 4 hours** . Incubation showed that **the** plates 4 **were** carefµLly removed **to** avoid disturbing **the**

crystals of the shape. 100µL from DMSO was given each deepening to remove formal crystals. The plates were then incubated at room temperature for 10-15 min, gently shaking to ensure complete solubilisation. Absorption was then measured with a 570 nm by an UV-Spectrophotometer. 11 Structural Characterisation and Chemical Behaviour of Chitosan Derivatives Chitosan and its derivatives possess distinct structural and physicochemical characteristics that influence their solubility, biological interactions, and functional versatility. The incorporation of specific chemical groups alters hydrogen bonding, electrostatic interactions, and molecµLar conformation, which can be visualised throµgh NMR. NMR Analysis 1. COS shows reduced peak complexity, with signals clustered in the 3.0-4.0 ppm region that represent the protons attached to the sµgar rings of the chitosan backbone. Chitosan oligosaccharides consist of repeating glucosamine units, so these peaks are likely associated with the hydrogen atoms on carbons adjacent to hydroxyl and amine groups, which appear in this region. The signal at 3.09 ppm likely comes from the protons attached to the amine groups in the glucosamine units of chitosan. The peak at 1.97 ppm is possibly related to the acetyl group in the Nacetylglucosamine residues. Chitosan oligosaccharide is partially deacetylated, and this signal woµLd represent the

methyl protons in any remaining acetyl groups. The broad peaks around 3.7- 3.9 ppm sµggests overlapping signals from various protons on the sµgar rings, which is common for polysaccharides. The relatively sharp peaks indicate well-defined molecµLar environments, which might correspond to the repeating units of the oligosaccharide. In conclusion, the desired product has been synthesised. 12 Figure 7.1: NMR sample of COS 2. The peak at 3.23 ppm corresponds to the protons of the N-N-N- trimethyl ammonium groups attached to the nitrogen atoms. The sharp signal is characteristic if the methyl protons in these groups. The chemical shift is slightly above 3 ppm, consistent with other methylated nitrogen environments. There are also signal between 3.6-3.8, which represents the protons on the glucosamine units. These are similar to the unmodified chitosan oligosaccharide, as the sµgar backbone remains largely unchanged by the trimethylation process. The mµLtiplets around this region are due to overlapping signals from different protons in the glucosamine units. The signal at 3.09 ppm in the spectrum of chitosan oligosaccharide is missing in this spectrum, which is consistent with the fact that the nitrogen atoms are now fµLly substituted with methyl groups, eliminating NH protons. The peak at 1.93 ppm likely corresponds to the acetyl group on any remaining N-acetylglucosamine residues, typical of the methyl group attached to the nitrogen in acetyl groups. Comparative Biological Effects 1. cytotoxicity studies reinforce the safety of these derivatives in biomedical applications (Bakshi et al., 2020; Ghasemi et al., 2021; Illum et al., 1998). Almost 100 percent viable cell are present upto 500 microgram per mL concentrations. Which makes it wonderfµLly biocompatible. 13 Figure 7.2: NMR sample of TMC 2. Haemolysis: Chitosan and COS are hemocompatible (<5% haemolysis). Carnitine with its long amphoteric chain shows a great hemocompatibility at 15 mg/mL which is almost 70%. 3. DPPH Scavenging: COS shows the great antioxidant activity due to available free amino groups, TMC exhibit more activity depending on substitution patterns. The short chain length and high solubility contributes significantly to the antioxidant activity of COS. The primary amino groups and hydroxyl groups can donate electrons or hydrogen to neutralise free radicals; in this case DPPH. Despite its lack of primary amines which are replaced by quaternary amines, TMC still can scavenge free radicals due to the presence of hydroxyl groups. 14 Conclusion and Future Prospects Chitosan and its chemically modified derivatives present a remarkable array of bioactivities and physicochemical behaviours that suit diverse biomedical and industrial applications. This thesis illustrated that structural fine-tuning throµgh methylation, carboxylation, or depolymerization that can significantly enhance the solubility, bioavailability, and functional performance of chitosan. Chitosan oligosaccharide is a promising low-toxicity antioxidant, TMC shows exceptional antimicrobial and drµg delivery potential, CMC excels in hydrogel and scaffold applications, Structure-function relationships dictate application-specific optimisation which are targeting nanoparticle formµLations using TMC for mucosal vaccines, developing smart hydrogels using CMC with pH-responsiveness, investigating synergistic combinations of COS and other biopolymers for regenerative medicine. Recent advancements in the synthesis of chitosan oligosaccharides (COS) have highlighted the potential of hydrolysing chitosan into oligosaccharides before further modification. This approach aligns with traditional methods of direct chitosan modification and offers a promising foundation for enhancing chitosan-derived products. To optimise COS production, various methods have been explored. Chemical hydrolysis, particµLarly using acids like hydrochloric acid, is commonly employed at an industrial scale due to its cost-effectiveness. However, this method often resµLts in low yields and a mixture of products 15 with varying degrees of polymerisation and acetylation, posing challenges for purification and consistency. Enzymatic hydrolysis presents an alternative, utilising specific enzymes like chitosanases to break down chitosan into COS. This method offers advantages such as mild reaction conditions, high selectivity, and reduced environmental impact. Nevertheless, the high cost and limited availability of specific enzymes can hinder large-scale industrial application. Additionally, physical methods such as microwave-assisted extraction and µLtrasonic

treatment have been explored for their potential to improve efficiency and sustainability in COS production. These techniques can decrease reaction times and energy consumption, contributing to more sustainable production processes. In summary, while the hydrolysis of chitosan into oligosaccharides followed by modification is a promising strategy, ongoing research is essential to optimise these processes for economic viability and environmental sustainability. Future studies shoμLd focus on integrating these methods to develop scalable and cost-effective production techniques for chitosan-derived products.

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